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SEPARATION OF α-, β- and γ-Casein[1]

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Casein was long considered to be a pure protein. However, it became apparent from the studies of Linderstrøm-Lang (6) and others that modification of this view was necessary. The electrophoretic investigation of Mellander (9) demonstrated that casein is composed of at least three components, which he designated α-, β- and γ-casein in the order of their decreasing mobilities. It was apparent from previous attempts to fractionate casein, as well as the constancy of its composition, that the separation of the electrophoretic components would be difficult. Warner (12), who devised the first chemical method for separating α- and β-casein, has reviewed previous methods for fractionating casein. His method depends on differences in the solubility of α- and β-casein in water at pH 4.4 and 2° C. This method, which requires repeated precipitations from dilute solutions, becomes tedious when large amounts of the purified casein components are desired. A method for the separation of γ-casein, based on its solubility in 50 per cent alcohol, has been reported recently by Hipp *et al.* (5).

This paper describes two methods suitable for separating the three components of casein in quantity. The first method is based on differences in their solubility in 50 per cent alcohol in the presence of salt, as well as in water, with changes in temperature and pH. The second method is based on their solubility in aqueous urea solutions at the isoelectric point of casein. The problem of devising a method for separating the three caseins involves finding conditions where their interactions are reduced.

EXPERIMENTAL

Acid casein was prepared from unpasteurized skimmilk by acidification, as described by Hipp *et al.* (5). When not used immediately, the wet casein was stored with toluol at about −20° C. The progress of fractionation was followed electrophoretically, according to the Tiselius method. The method of calculating areas and mobilities was the same as that described by Warner (12). The electrophoretic pattern, shown in figure 1, a, indicates that unfractionated casein contains about 75 per cent α-casein, 22 per cent β-casein and 3 per cent γ-casein.

Measurements of pH in 50 per cent alcohol were made in the usual manner with the glass electrode. Although no absolute significance is attached to the pH values in 50 per cent alcohol, procedures based on these measurements are reproducible. The point of minimum solubility of unfractionated casein is about

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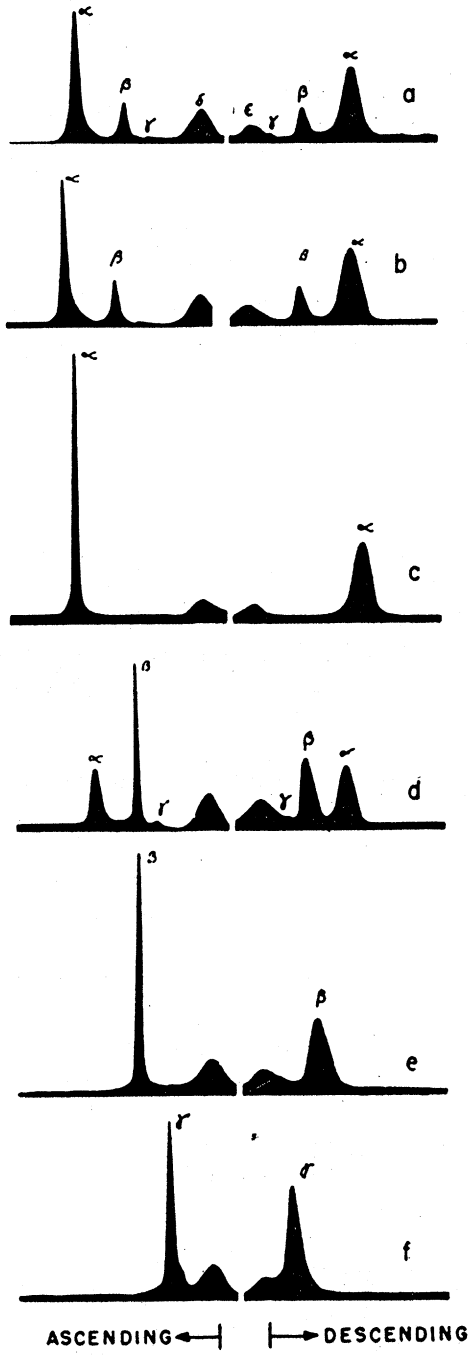

FIG. 1. Electrophoretic patterns obtained in a veronal buffer at pH 8.4 with an ionic strength of 0.1, containing 0.05 *N* NaCl, protein concentration 1%, at field strength of 4.33–4.86 volts/cm. after electrophoresis for 3 hr. (a) Unfractionated casein; (b) fraction B; (c) α-casein; (d) fraction C; (e) β-casein; (f) γ-casein.

pH 5.7 in 50 per cent alcohol, as compared with pH 4.7 in water. Fractionations were carried out at room temperature, (20–28° C.) unless otherwise stated.

Fractionation of casein in 50 per cent alcohol

Exploratory experiments showed that the solubility of isoelectric casein in ethyl alcohol-water solutions was greatest when the alcohol concentration was about 50 per cent. It was demonstrated also that the presence of salt is important in separating the electrophoretic components of casein with alcohol. As a preliminary step, casein was separated into three fractions, designated A, B and C, by adjusting the pH and the temperature of an alkaline solution of casein in 50 per cent alcohol containing 0.2 *M* ammonium acetate. Approximately 1,000 g. (dry weight) of wet casein were suspended in water and dissolved by the slow addition of 0.1 *N* NH_4OH to give a 6.6 per cent solution of casein of pH 7 when adjusted to a volume of 15 l. by adding water. Ammonium acetate (462 g.) then was added, making the solution 0.4 *M*, and an equal volume of absolute alcohol was added slowly, with stirring.

Fraction A. The alcoholic casein solution was adjusted to pH 6.5 by the slow addition of 1 *N* acetic acid in 50 per cent alcohol, precipitating fraction A. After standing for several hours or overnight, the precipitate was removed by filtration through 50-cm. fluted filter papers. The precipitate, amounting to 30 per cent of the total casein, contained 92 per cent α-casein and 8 per cent β-casein. It also contained the proteolytic enzyme of casein (13).

Fraction B. The filtrate obtained from fraction A was adjusted to pH 5.7 by adding 2 *N* acetic acid in 50 per cent alcohol. Stirring was continued for 1 hr. in order to collect the gummy precipitate. After standing for 3 hr. or overnight, the insoluble precipitate, fraction B, was filtered on large fluted papers. This fraction, amounting to 39 per cent of the total casein, contained 80 per cent α-casein and 20 per cent β-casein.

Fraction C. The protein in the filtrate from fraction B was precipitated by cooling to 2° C. An alternate, though less desirable method, is to dilute with an equal volume of water at room temperature. This fraction, amounting to 31 per cent of the total casein, contained 44 per cent α-casein, 50 per cent β-casein and 6 per cent γ-casein.

Preparation of α-casein. Electrophoretically pure α-casein may be prepared from either fraction A or B. It was prepared more easily from fraction A, but, when prepared from this fraction, it contained the proteolytic enzyme and when dissolved gave a slightly turbid solution.

In preparing α-casein from fraction A, the wet precipitate was suspended in water and dissolved by adding 0.1 *N* NH_4OH to give a solution of pH 7.2. The protein concentration was adjusted to about 6 per cent by diluting with water. A volume of absolute alcohol equal to that of the water used then was added, increasing the apparent pH of the solution to 7.7. Electrophoretically, pure α-casein was precipitated by the slow addition of a solution of 2 *M* ammonium acetate in 50 per cent alcohol until the pH was 7.2; at this point a definite granular precipitate formed. Usually, about 25–30 ml. of ammonium acetate

solution were required for each liter of solution. The precipitate was removed by centrifugation and washed at least three times with 50 per cent alcohol containing 0.07 *M* ammonium acetate. To make α-casein isoelectric, the precipitate was dissolved with dilute NH_4OH and reprecipitated with dilute acetic acid at pH 4.7 and washed free of salt. The yield of dry α-casein was about 15 per cent, based on the weight of the unfractionated casein used.

This procedure did not give pure α-casein when applied to fraction B. Pure α-casein could be prepared, however, by dissolving fraction B in borax and precipitating it in 50 per cent alcohol at pH 5.7 in the presence of 0.15 *M* NaCl. Two kg. of wet fraction B precipitate (800 g. dry weight) were suspended in water and dissolved by the addition of 280 g. of borax and adjusted to 13 l. with water, giving a 6 per cent protein solution at pH 8. NaCl (228 g.) and 13 l. of absolute alcohol were added, making the solution 0.15 *M* NaCl and approximately 50 per cent alcohol. The protein then was precipitated at pH 5.7 by adding 2.1 l. of 0.5 *N* HCl in 50 per cent alcohol. After standing overnight, the supernatant was removed by decantation. The sticky precipitate was washed with 2 l. of 50 per cent alcohol containing 0.15 *M* NaCl. This fractionation was repeated twice, with the exception that the volumes used were reduced to one-half the preceding volume. A yield of 285 g. of α-casein was obtained from 800 g. of fraction B, or about 15 per cent, based on the original casein used.

Preparation of β-casein. β-casein is concentrated in fraction C. This fraction contained about equal parts of α- and β-casein and all the γ-casein. Pure β-casein was separated from the other components by utilizing the difference in solubility of the three components in water at 2 and 25° C. (12). Fraction C (1 kg.) was suspended in water and dissolved with a minimum quantity of dilute NaOH and diluted to a protein concentration of about 1 per cent. After it was cooled to 2° C. cold dilute HCl was added dropwise to the solution, with constant stirring, until the pH was 4.5. The precipitate formed under these conditions was largely α-casein, which was removed by filtration at 2° C. Crude β-casein was precipitated from the filtrate by warming to 32° C. and it then was removed by filtration, leaving most of the γ-casein in the filtrate. This filtrate, as well as the second filtrate obtained in a similar manner, was used for preparing γ-casein.

After two extractions at 2° C. and pH 4.5, the insoluble fraction was free of γ-casein and was used as a source of β-casein. It was dissolved to make a 1 per cent solution and then was reprecipitated five times at pH 4.3 and 2° C. A total yield of 115 g., containing 98 per cent β-casein, was obtained by warming these filtrates to 32° C. Three further extractions of the insoluble residue at pH 4.0 yielded 160 g. of 90 to 95 per cent β-casein. Pure β-casein was obtained by reworking the fractions containing more than 90 per cent of this component. The α-casein impurity was removed by precipitation from a 0.1 per cent solution at pH 4.4 and 2° C. The filtrate was adjusted to pH 4.9, and β-casein was precipitated by warming to 32° C. After dissolving and filtering, the product was precipitated at its isoelectric point of pH 4.9 and washed free of salt. It is advantageous to use acetone followed by ether in drying the product, rather than

alcohol, since β-casein is soluble in alcohol-water solutions. A total of 166 g. of electrophoretically pure β-casein (figure 1, e) was obtained.

Preparation of γ-casein. The first two filtrates obtained in preparing crude β-casein by precipitating from solution at 32° C. were used for preparing γ-casein. Electrophoretic patterns indicated that this fraction contained 44 per cent γ-casein, 12 per cent β-casein, and 44 per cent of a component of casein moving more slowly than γ-casein. The total protein was precipitated from these filtrates by adding enough NaCl to adjust to 0.5 *M* and removed by filtration. The precipitate was dissolved from the filter paper in dilute NaOH and reprecipitated from a volume of 1500 ml. in the presence of 0.5 *M* NaCl by the addition of acid until the pH was 5.0. The precipitate, containing about 30 g. protein, was collected by means of centrifugation. It then was dissolved in dilute NaOH and dialyzed in the cold to free it of NaCl. The crude γ-casein solution, with a volume of 200 ml., was adjusted to pH 6.4 by addition of 0.01 *N* HCl at room temperature. The protein was largely precipitated as a sirup. Thirteen g. (dry basis) of the sirupy precipitate were extracted three times with 500 ml. of 50 per cent alcohol at 35° C. and cooled to room temperature. The insoluble material was removed by centrifugation. After addition of 500 ml. of 50 per cent alcohol to clarify the combined supernatant, the product was precipitated by cooling to 2° C. After standing for a day, the precipitate was removed by centrifugation at 2° C. γ-Casein then was dissolved in 2 l. of 50 per cent alcohol at room temperature and reprecipitated at 2° C. as before. The precipitate was removed by centrifugation at 2° C., and washed with 20 volumes of acetone. The product then was dried with a mixture of equal volumes of acetone and ether at room temperature. A yield of 6 g. of pure γ-casein having the electrophoretic pattern shown in figure 1, f was obtained.

Fractionation of casein by means of aqueous urea solutions

Whole casein, such as that freshly prepared from skimmilk or the dried product, when dissolved in concentrated aqueous urea solutions, can be separated into its components by merely adding water in suitable amounts (figure 2, a, b and c). Thus, when the concentration of urea was reduced to 4.6 *M* by the addition of water, α-casein became insoluble. After the removal of residual α-casein by the addition of water, β-casein was precipitated from the filtrate by making the urea concentration 1.7 *M*. γ-Casein remained in solution and was removed by further dilution with water or by the addition of $(NH_4)_2SO_4$. The process is operable at ordinary room temperature.

The following example illustrates the method of separating the components of casein by means of urea solutions:

Fifteen hundred g. of wet casein (400 g., dry weight) were dissolved by addition of 1,500 g. of urea and water, making a total volume of 3.75 l. and giving a urea concentration of 6.6 *M*.

α-Casein. The solution was warmed to room temperature, and 1.65 l. of water were added slowly, with stirring, to the casein-urea solution, making the concentration of urea 4.63 *M*. The precipitate formed under these conditions was

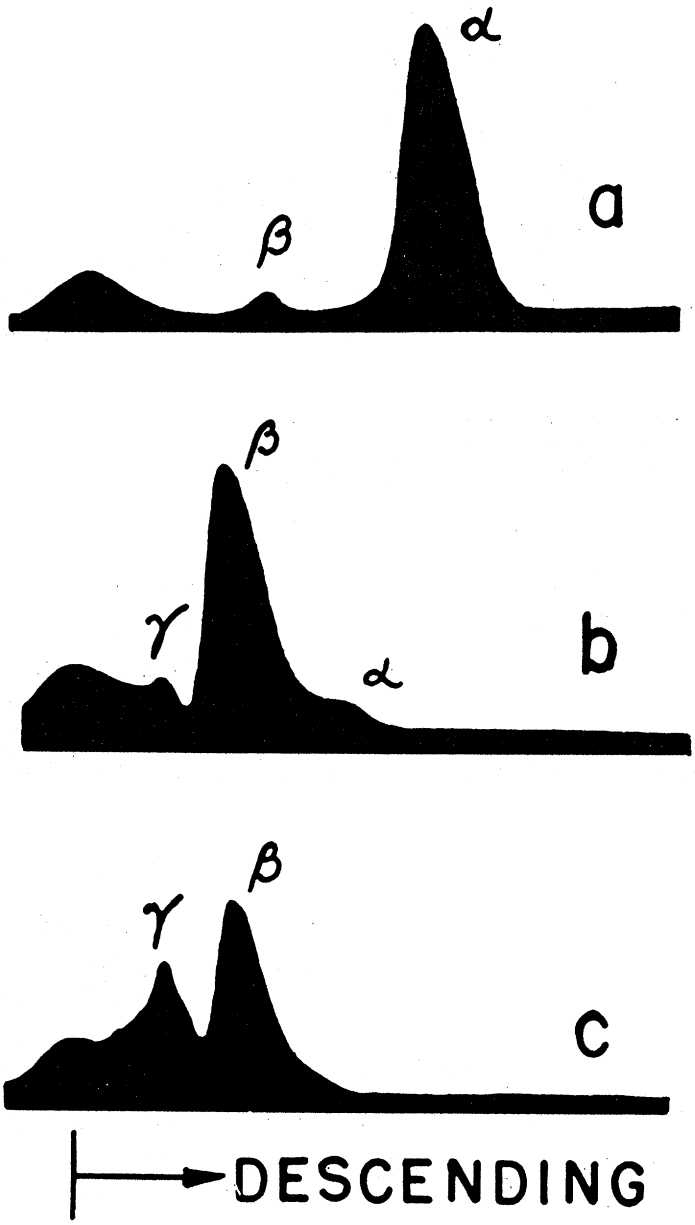

FIG. 2. Electrophoretic patterns obtained in a veronal buffer at pH 8.4 with an ionic strength of 0.1, containing 0.05 *N* NaCl, protein concentration 1%, at a field strength of 4.56–5.05 volts/cm., after electrophoresis for 3 hr.; (a) Insoluble in 4.6 molar urea; (b) soluble in 3.3 and insoluble in 1.7 molar urea; (c) soluble in 1.7 molar urea.

largely α-casein (figure 2, a), which was removed by centrifugation. The crude α-casein was further purified by dissolving it in 1,500 ml. of 6.6 *M* urea containing 15.9 g. of NaCl. α-Casein was precipitated by the addition of 1,500 ml. of water.

Further impurities were removed by washing the precipitate with a 4.7 *M* urea solution. In order to remove a trace of β-casein, the product again was dissolved in urea and reprecipitated as before. Urea was removed from the product by washing with an excess of water. After drying, 180 g. of pure α-casein were obtained, a calculated yield of 60 per cent, based on the α-casein content of the whole casein used. This α-casein contained a small amount of proteolytic activity (13).

β-Casein. The filtrate, obtained after the removal of the precipitated α-casein at 4.63 *M* urea, was diluted to 3.3 *M* urea by the addition of water. The small insoluble precipitate obtained here was a mixture of α- and β-casein in about the proportion in which they occur in unfractionated casein; it was removed by centrifugation and discarded. The portion soluble in 3.3 *M* urea was diluted to 1.7 *M* urea, and the pH was adjusted to 4.7 by addition of about 20 ml. of 0.1 *N* HCl. Seventy-eight g. of crude β-casein, (figure 2, b) were precipitated. The crude β-casein was purified by dissolving it in 2 l. of 4.6 *M* urea and fractionating by dilution with water. Again, the portion soluble is 3.3 *M* urea but insoluble in 1.7 *M* urea contained the purest β-casein. By reprecipitation in this manner, the remaining small amount of α-casein was removed. Thirty-two g. of pure β-casein were obtained, a yield of 40 per cent, based on the total β-casein in the whole casein used. By reworking the material insoluble in 3.3 *M* urea obtained in purifying β-casein, the yield of β-casein can be increased.

γ-Casein. The casein soluble in 1.7 *M* urea, obtained from the supernatant of the crude β-casein precipitate, was precipitated by adding solid $(NH_4)_2SO_4$ to the solution until the concentration of $(NH_4)_2SO_4$ was 1.6 *M*. The casein was precipitated completely and separated by filtration. From 20 to 30 g. of the dried salt-free casein were obtained. This fraction contained 40 per cent γ-casein contaminated with some impurity and 60 per cent β-casein (figure 2, c). The γ-casein was further concentrated by dissolving this material in 250 ml. of 2 *M* urea and removing impurities by warming the solution to 60° C. and diluting to 1 *M* urea. The precipitate formed, which was removed by centrifugation, contained large amounts of β-casein. The γ-casein fraction was precipitated from the supernatant by adding solid $(NH_4)_2SO_4$. By repeating this procedure of dissolving and reprecipitating the portion insoluble in 1.0 *M* urea, the yield of γ-casein can be increased. The material precipitated with $(NH_4)_2SO_4$ contained 80 per cent γ-casein and 20 per cent β-casein. Pure γ-casein was obtained by dissolving this product in dilute NaOH and precipitating the impurities at pH 4.7 and 2° C. The supernatant was adjusted to pH 5.8 at 2° C., and γ-casein was precipitated by warming to 30° C. About 3 to 4 g. of pure γ-casein were obtained, a yield of 25 per cent, based on the γ-casein in the starting material.

DISCUSSION

Several methods have been described previously for fractionating casein by means of alcohol. The extensive method of Linderstrøm-Lang (7) is based on separations in acid-alcohol solutions at relatively high temperatures. Although no evidence was given for the homogeneity of fractions prepared by the acid-

alcohol method, the variations in phosphorus content of the fractions indicated separation. It appears likely that Linderstrøm-Lang's fraction K_1, containing 0.1 per cent phosphorus and amounting to 3 per cent of the original casein, contained a large amount of γ-casein. No attempt was made to prepare γ-casein by Linderstrøm-Lang's procedure. It was found (12), however, that the separation of α- and β-casein by his method was slight, as indicated by electrophoresis.

Groh *et al.* (4) fractionated casein from phenol, urea-alcohol and 70 per cent alcohol solutions. Their fractions were characterized by the tyrosine, tryptophane and phosphorus contents, as well as by optical rotation. The values for the tyrosine and tryptophane contents of their K_1 fractions are in good agreement with Gordon *et al.* (2) for α-casein. Their values for the specific rotation of their K_1 and K_2 fractions also are in good agreement with the values reported by Hipp *et al.* (5) for α- and β-casein, respectively. However, their values for the phosphorus content of fractions K_1 and K_2 indicate incomplete separation when compared with the phosphorus content of α- and β-casein. Our experience with the ammoniacal 70 per cent alcohol method of Groh *et al.* (4) indicates that by their method pure α-casein can be prepared from their K_1 fraction in small yields, but that their K_2 fraction contained only about 80 per cent β-casein.

The present method of separating the components of casein by alcohol depends in part on variations in the solubility of the components in 50 per cent alcohol. The efficiency of separation, however, was greatly increased by the presence of salt. Both these solubility effects are consistent with the amino acid composition of α- and β-casein, as determined by Gordon *et al.* (2). They found that α-casein contained 548 polar groups and 341 nonpolar groups, whereas β-casein contained 465 polar groups and 442 nonpolar groups per 10^5 g., showing that β-casein is more nonpolar than α-casein; consequently, both alcohol and salt are effective in their separation. The amino acid analyses of γ-casein are incomplete as yet, but the proline content was found to be 17 g. per 100 g. protein, as compared with 15.1 g. in β-casein and 7.47 g. in α-casein (3).

The high proline and low phosphorus contents of γ-casein are consistent with its high solubility in alcohol. The solubility of α-, β- and γ-casein in water and 50 per cent alcohol has been reported previously (5). γ-Casein and β-casein are much more soluble in water and in 50 per cent alcohol than is α-casein. Moreover, β- and γ-casein are at least fifteen times more soluble in water at 2.5 than at 25° C. These similarities in properties of β- and γ-casein indicate similarities in structure.

Separation of the electrophoretic components of casein by means of aqueous urea was particularly effective. An inexpensive purified grade of urea was used. The separations can be made from concentrated casein solutions, making preparation of the components in quantity convenient. Burk and Greenburg (1) found that molecular weight of unfractionated casein in 6.6 *M* urea is 33,600. Since this value is much lower than the value of 75,000 to 100,000 reported by Svedberg *et al.* (11), it has been thought that urea splits the casein molecule (10). The pure components of casein separated by means of urea have the same composition and properties (table 1) as when separated by changes in pH or alcohol,

indicating that urea does not split the casein molecule. Groh *et al.* (4) devised a method of separating casein by means of urea and alcohol. The use of aqueous urea solutions by the method herein described is less complicated and results in the separation of electrophoretically pure components.

It was noted by Warner (12) that the electrophoretic pattern of the rising boundary of α-casein frequently showed two peaks at pH values alkaline to the isoelectric point. In the present work, the rising boundary of α-casein showed only one peak. This difference can be accounted for by the fact that the casein used by Warner was obtained from the milk of one or two cows, whereas the casein herein described was from mixed milk obtained from a large number of animals. McMeekin *et al.* (8) have shown that the electrophoretic pattern of the α-casein component from individual cows occasionally has two peaks. The reason

TABLE 1

Composition and properties of the components of casein prepared by various methods

Method of preparation	N[a, b]	P[a]	S[a]	Mobility[c]	Specific[d] rotation	Intrinsic[e] viscosity
	(%)	(%)	(%)	(u)	$([\alpha]_D^{25})$	($[\eta]$)
Isoelectric, 2° C.						
α	15.56	0.982		−6.64	− 88.2	0.105
β	15.39	0.602				
Aqueous alcohol						
α	15.58	0.99	0.72	−6.75	− 90.5	0.100
β	15.33	0.55	0.86	−3.05	−125.2	0.147
γ	15.40	0.11	1.03	−2.01	−131.9	0.095
Aqueous urea						
α	15.57	0.98		−6.50	− 87.4	0.105
β	15.47	0.64		−3.15	−124.6	0.145
γ	15.59			−2.04		

[a] On a moisture-free basis.

[b] We are indebted to Mary Jane Welsh for making the nitrogen determinations.

[c] In veronal buffer at pH 8.4, $\mu = 0.1$, containing 0.05 *N* NaCl, 1% solution, calculated from descending boundary in $cm^2\ volt^{-1}\ sec^{-1} \times 10^5$.

[d] Determined on 1% solution in pH 8.4 veronal buffer, $\mu = 0.1$. A value of −105 was obtained for unfractionated casein.

[e] Determined in 0.05 *N* NaCl at pH 6.4 to 6.9, (concentration in g./100 ml. of solution). These values for intrinsic viscosity are an indication of molecular size.

for this difference in the casein from individual cows is obscure. No difference was noted in the composition of α-casein with one electrophoretic peak and that of α-casein with two peaks.

SUMMARY

Two methods are described for separation of the electrophoretic components of casein. In the first method, the separation is accomplished by precipitation from 50 per cent alcohol solutions of casein by means of variations in temperature, pH and ionic strength and by isoelectric precipitation from water.

The second method is based on the solubility of the casein components in aqueous urea. The urea method is relatively simple and gives products with the same composition and properties as those obtained by the pH and also the 50 per cent alcohol methods.

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